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PLANT MICROBE INTERACTIONS

Competitiveness of Diverse *Methylobacterium* Strains in the Phyllosphere of *Arabidopsis thaliana* and Identification of Representative Models, Including *M. extorquens* PA1

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Abstract Facultative methylotrophic bacteria of the genus *Methylobacterium* are consistently found in association with plants, particularly in the phyllosphere. To gain a better understanding of the mechanisms underlying the dispersal and occurrence of *Methylobacterium* on plants, diverse strains were isolated, identified, and studied with regard to their competitiveness on the model plant *Arabidopsis thaliana*. As a basis for this study a comprehensive collection of *Methylobacterium* isolates was established. Isolates were obtained from five different naturally grown *A. thaliana* populations and diverse other plant genera at these and further sites. They were classified using automated ribosomal internal spacer analysis (ARISA) and a representative subset was identified based on 16S rRNA gene sequence analysis. A comparison of their ARISA patterns with those generated based on a cultivation-independent approach from the same sampling material confirmed that the isolates were abundant colonizers of the studied plants. In competition experiments, colonization efficiency of the strains was found to be linked to phylogeny, rather than to the geographical origin or plant genus from which they were isolated. The most competitive colonizers were related to the species *Methylobacterium tardum* and *Methylobacterium extorquens*. Higher cell numbers were observed in the phyllosphere of *A. thaliana* when a mixture of different strains was applied relative to inoculation with only one strain, suggesting partial niche heterogeneity. Based on the results of the competition experiments, representative strains with different colonization efficiencies were selected, which will serve as models in future studies aiming at a better understanding of plant colonization by this bacterial genus. Among them is the meanwhile genome-sequenced strain *M. extorquens* PA1, which represents a competitive species of plant colonizers with a broad dispersal. This strain was characterized in more detail including physiological, morphological, and chemotaxonomical properties.

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Introduction

Members of the genus *Methylobacterium* are pink-pigmented facultative methylotrophs that occur ubiquitously in nature; they have been detected in soil, dust, freshwater, sediments, and in the air [17]. Moreover, they are consistently found in association with plants, in particular as epiphytic and endophytic leaf colonizers [6, 9, 28], but also in association with plant roots [2, 38, 53]. Diverse *Methylobacterium* strains have been isolated from plants, especially from the aerial part, termed the phyllosphere [25, 27, 36, 37, 44, 61], or detected by cultivation-independent methods [4, 7, 21, 23, 32, 50]. The characterization of *Methylobacterium* communities on plant leaves by cultivation-independent methods revealed clear differences in the composition at different sampling sites and between some of the different plant species within the sampling sites [27, 28]. Furthermore, the complexity of *Methylobacterium* communities has been reported to be

dissimilar on different plant species grown at the same site [27, 49].

Insight into the lifestyle of *Methylobacterium* on plant leaves was obtained in recent studies, which showed that methylotrophy and general stress response are important traits of *Methylobacterium* for successful plant colonization [14, 15, 58]. However, these traits are apparently shared among the members of the genus and do not explain the observed variation in the *Methylobacterium* community composition on distinct plant species and at different sites. The factors that affect the formation of *Methylobacterium* communities on plants remain to be identified, as well as the bacterial traits that respond to these factors.

Studies based on defined model systems with reduced complexity will be important in elucidating the molecular mechanisms underlying community structures. Representative models will also be helpful in studies leading to a better understanding of the ecological role of *Methylobacterium* in the phyllosphere. *Methylobacterium* may be of particular importance with respect to methanol recycling. Methanol is emitted as a major volatile organic compound by plants with an estimated annual release of 100 Tg [12]. Additionally, *Methylobacterium* may influence plant health and development. Certain plants have been shown to respond to the presence of *Methylobacterium* by accelerating growth [1, 20, 31, 33, 51], a response that is presumably mediated by bacterially produced phytohormones such as auxins and cytokinins [29, 41, 59]. As a prerequisite for isolate-based studies, the dispersal of plant colonizing *Methylobacterium* strains within the genus was studied and the competitiveness of diverse strains was evaluated on *Arabidopsis thaliana*, the best studied plant model [56].

Methods

Isolation and Cultivation of Methylobacterium Strains Isolates of the genus *Methylobacterium* were obtained from diverse plants at eight different sampling sites. The sites harboring *A. thaliana* populations were located in Spain (A1–A5). Additional samples were taken from sites in southern France (M1–M3). More details about the sites are published in Knief et al. 2010 [28]. In that study, the very same plant material was analyzed with respect to *Methylobacterium* community composition by automated ribosomal internal spacer analysis (ARISA), and the population size was determined by spotting 5-μl aliquots of serial dilutions, prepared from macerated plant material, on mineral salts medium agar plates supplemented with 120 mM methanol. This medium was applied as such and in a fivefold diluted version. In the present study, the plated dilution series were used to isolate methylotrophic bacteria. All colonies with different morphologies, i.e., of different size, pigmentation nuances, and shape, were picked per plant species and site from both, the diluted and undiluted medium, and restreaked until pure cultures were obtained. The purity of the isolates was verified on nutrient agar plates containing (per l): 5 g peptone, 3 g beef extract, 15 g agar (pH 6.9). Moreover, isolates were collected from the air on two 12×12 cm square plates filled with methanol-supplemented medium, which were deposited on the ground at the sampling site for 15 and 30 min, respectively. Between 1.9 and 7.7 colony forming units [CFU] cm^{-2} h^{-1} were obtained on the plates by this method at the different sampling sites.

Strain Classification by ARISA and Strain Differentiation by BOX-PCR Single colonies were picked from agar plates, resuspended in 100 μl of 0.05 M NaOH solution, and lysed by incubation at 95°C for 10 min. One-microliter aliquots of these suspensions were added to PCR assays, which had a total volume of 20 μl. ARISA was performed as described previously [27]. BOX-PCR fingerprints were generated according to the protocol of Rademaker et al. [48]. The BOX-PCR assay contained 2 μl of 10-fold PCR buffer (Invitrogen), 7.5 μM $MgCl_2$, 1.25 mM of each dNTP (Invitrogen), 100 μg BSA ml^{-1} (Roche Diagnostics), 2 μM of primer BOX A1R, 1.6 U Platinum *Taq* DNA polymerase (Invitrogen), and 1 μl of template DNA. The BOX-patterns were compared using the GelCompar II software (Applied Maths, Sint-Martens-Latem, Belgium).

16S rRNA Gene and Internal Transcribed Spacer 1 (ITS1) Sequence Analysis The central part of the 16S rRNA gene was sequenced using primer 533f as described previously [27]. Nearly complete 16S rRNA gene sequences were generated for representative strains with different partial 16S rRNA gene sequences (those with more than 2 bp difference in their partial sequence). The ITS1 and the nearly-complete 16S rRNA gene were PCR amplified and sequenced as described in Knief et al. [27]. Sequence assembly was performed with Vector NTI (Invitrogen). Phylogenetic sequence analysis including ITS1 sequence alignment based on ClustalW was performed using the ARB [35] and Phylip 3.68 software packages [10].

Competition Experiments In Planta A. thaliana seeds were sterilized with 2.4% hypochlorite as described [14]. The seeds were then added to a bacterial suspension and incubated at room temperature for at least 30 min. The bacterial suspensions were prepared in 2.5-fold diluted mineral salts medium without carbon source. For the first competition experiment 2 ml of cell suspension were prepared by resuspending half an inoculation loop of cell material per *Methylobacterium* strain, cultured on 30 mM succinate plates for 5 days. Each strain was used in two

different combinations with four other strains. Each combination of strains was tested in three independently prepared suspensions to account for possible slight variations in the composition of the mixtures due to the preparation method. Three seeds and 1 ml of each cell suspension were pipetted onto a peat pellet (Jiffy-7, Ø= 44 mm, Jiffy Products International AS, Norway) to grow the plants. An indigenous *Methylobacterium* population could not be detected in these peat pellets (data not shown). The plants were grown in the greenhouse at 23°C with a 12.5 h light period for 3 weeks. The boxes with the peat pellets were sealed with cling film during the first week of incubation to maintain humid conditions during seed germination.

The second competition experiment was performed in the same way as the first, but cell mixtures were prepared with four different strains in three independently prepared suspensions, and each suspension was applied to two peat pellets. Each strain was tested in multiple strain combinations to evaluate competitiveness with all other strains. For the third competition experiment, one loop of cell material from methanol grown cultures was used to prepare cell suspensions consisting of three different strains. Three independently prepared suspensions per strain combination were applied to two peat pellets.

The second competition experiment was additionally performed under gnotobiotic conditions in microboxes (Combiness, Nazareth [Eke], Belgium) as described before [14]. Sterilized seeds were placed on solidified Murashige and Skoog medium [39] and 5 µl of cell suspension was pipetted over each seed. Each suspension of mixed strains was used to inoculate eight seeds in one microbox. Moreover, inoculation experiments with individual strains (single inoculation) were performed. For this purpose, half a loop of cell material was resuspended in 1 ml of diluted medium, resulting in an OD_{600} of 0.6–0.9. Each strain was tested based on two independently prepared suspensions and each suspension applied to eight seeds. The subsequent analysis of the *Methylobacterium* cell numbers in the plant phyllosphere revealed that slight variations in the cell numbers of the inocula of the independently prepared suspensions did not affect the final cell numbers.

Identification of Competitive Methylobacterium Strains The competitiveness of the *Methylobacterium* strains was evaluated on seed-inoculated 3-week old plants. The aerial parts of all plants grown on the same peat pellet or of individual plants grown under gnotobiotic conditions were transferred into a weighed 1.5-ml reaction tube, and the plant weight was determined. Then, the plant material was macerated with a pellet pestle to access also endophytic cells, a fivefold dilution series was prepared, and a 5-µl aliquot of each dilution was spotted on two methanol-supplemented mineral salts medium agar plates. These dilution series were used to determine the number of colony forming units in the plant phyllosphere. In order to identify the most competitive strains, single colonies were picked from the highest dilutions, resuspended in NaOH solution, and the ITS1 was amplified using a *Methylobacterium*-specific primer and then sequenced with the nested primer 1492f. Generally, between eight and 12 colonies were analyzed by this method for each strain combination. For the third competition experiment the identity of single colonies in the dilution series was analyzed based on ARISA and strain-specific PCR. The strain-specific PCR was developed to distinguish between *Methylobacterium extorquens* strains. Primers PA1_5f (TGC TGT TTT AGG ATG GTC GGC AGC) and PA1_8r (CCG TCT GCT GCG CAA GGC GCT GGC AGA) were designed, which bind specifically to the ITS1 of *M. extorquens* PA1 (isolate 157). These primers were applied in a PCR assay that was described previously for the specific detection of *Methylobacterium* [27]. Stringency of the primers was achieved by using 3 mM $MgCl_2$ and an annealing temperature of 72°C for 1 min.

Statistical Analyses All statistical analyses were performed in SPSS version 16.0. To analyze potential relationships between the competitiveness of the isolates and their origin or phylogenetic placement, the performance of a strain was judged as competitive when it was identified as dominant colonizer in at least one of the two strain combinations tested in the first competition experiment. The competitive and non-competitive strains were listed in contingency tables. The distribution of strains in dependence of the sampling site, the host plant species, and phylogeny was analyzed based on Pearson's chi-square tests, respectively. Owing to the testing of multiple factors, the resulting *P* values were subjected to a Bonferroni correction. To analyze the dispersal of competitive strains with respect to their phylogenetic placement, closely related isolates were grouped together using the Dotur software package [54]. A cut-off of <1% sequence identity was used to delineate 16S rRNA gene clusters, resulting in the formation of seven groups, while a cut-off of <14% was used for the ITS1 data, resulting in 12 groups, which corresponded to a resolution slightly below the species level. The few groups that consisted of only one isolate (isolates 88, 411, and 189 for the ITS1 dataset and isolates 440 and 189 for the 16S rRNA gene dataset) had to be removed before the contingency tables were analyzed in order to fulfill the requirements for applying the chi-square test and to guarantee reliable results.

Nucleotide Sequence Accession Numbers The nearly-complete 16S rRNA gene sequences obtained during this

study and the ITS1 sequences have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers FN868933 to FN868961 and FN869377 to FN869532, respectively.

Results and Discussion

Classification and Identification of Methylobacterium Isolates A collection of 635 *Methylobacterium* isolates was obtained from the phyllosphere of *A. thaliana* and 25 other plant genera (Table S1). Additional 233 isolates were obtained from the roots, air, and surface soil at the sampling sites. Methylotrophic bacteria of other genera than *Methylobacterium* were not enriched and isolated from the phyllosphere samples. However, isolates related to the methylotrophic genera *Methylophilus*, *Methylopila*, and *Hansschlegelia* were obtained from the root samples (data not shown). *Methylobacterium* isolates were obtained from normal strength mineral salt medium and a fivefold diluted version of this medium, as the phyllosphere is assumed to be rather poor in nutrients [34]. However, a similar set of isolates was obtained from both media, as concluded from a comparative analysis of ARISA patterns. Nearly all isolates that were isolated on diluted medium grew better on undiluted medium.

Classification of the isolates based on their ARISA patterns allows resolution of diversity at least up to species level [27]. This classification method served as a basis for the selection of strains for identification based on partial 16S rRNA gene sequencing (≥814 bp). At least one isolate of each ARISA pattern type per sampling site was analyzed. The 386 sequenced isolates represented 29 different genotypes. The phylogenetic placement of these genotypes was analyzed further in relation to *Methylobacterium* type strains based on nearly full length 16S rRNA gene sequences, which were generated for one representative strain per genotype (Fig. 1). One-third of the genotypes were detected at more than four of the eight sampling sites (Table 1). These more widely distributed genotypes fell into four major clusters: (I) *M. extorquens* strains (represented by isolate 157), (II) strains closely related to *Methylobacterium adhaesivum* AR27^T (isolates 69, 108, 109, 139, and 256), (III) strains belonging to a separate sequence cluster (isolates 32 and 281), probably representing a novel species most closely related to *M. adhaesivum* AR27^T (97.7–98.0% sequence identity), and (IV) strains with the genotype represented by isolate 31, which is most closely related to *Methylobacterium mesophilicum* A47^T (99.2%). Isolates with very high sequence identity (> 99.5%) to known type strains were less frequently obtained, e.g., the genotypes represented by isolates 52, 88, 572, and 711, which show a 100% sequence identity to the type strains of *Methylobacterium radiotolerans*, *Methylobacterium tardum*, *Methylobacterium brachiatum*, and *Methylobacterium mesophilicum*, respectively. The isolates from the soil and air represented in most cases the same 16S rRNA genotypes as the phyllosphere strains isolated from the respective site (Table 1) and most of them had the same ARISA patterns (Table S1).

Evaluation of the Representativeness of the Isolates Based on ARISA data, we analyzed whether the isolates represented abundant phyllosphere colonizers of the plants from which they were isolated. The ARISA peaks of the isolates that were obtained from the phyllosphere were therefore compared to cultivation-independently retrieved ARISA patterns from the very same sampling material, which reflect the *Methylobacterium* community composition on the plants that were used to isolate *Methylobacterium* [28]. The ARISA patterns of 55% to 100% of the isolates were also detected in the cultivation-independent ARISA patterns retrieved from the plants at the respective site (Table 2). The overlap increased to >97% when the ARISA patterns of the isolates were compared to the ARISA patterns obtained from all sampling sites. This suggests that the majority of isolates represent abundant plant colonizers. Conversely, between 40% and 94% of the summarized relative peak height in the cultivation-independent ARISA pattern from individual plants corresponded to peaks seen in isolates from the respective site. Even more peaks in the cultivation-independent ARISA patterns could be explained when isolates from all sites were considered (≥89% of the total peak height). This comparison indicates that essentially all of the major ARISA groups that were detected based on the cultivation-independent analysis were successfully isolated. Thus, a strong cultivation-based bias during enrichment and isolation seems to be an unlikely explanation for the higher frequency with which certain *Methylobacterium* genotypes were isolated in this study.

Instead, these frequently obtained genotypes may indeed represent common phyllosphere colonizers. This can be further substantiated by the observation that their 16S rRNA gene sequences were in a number of cases closely related or identical to 16S rRNA gene sequences from phyllosphere-inhabiting bacteria detected in other studies (Fig. 1). These include genotypes related to *M. extorquens* and genotypes in the sequence cluster that is distantly related to *M. adhaesivum*. Moreover, we isolated several strains with 16S rRNA gene sequences identical to these genotypes from herbal plants at a sampling site in southern France, and from mosses collected in northern Germany (additionally listed in Table S1).

To analyze the distribution of plant-associated strains within the genus *Methylobacterium* more broadly, around

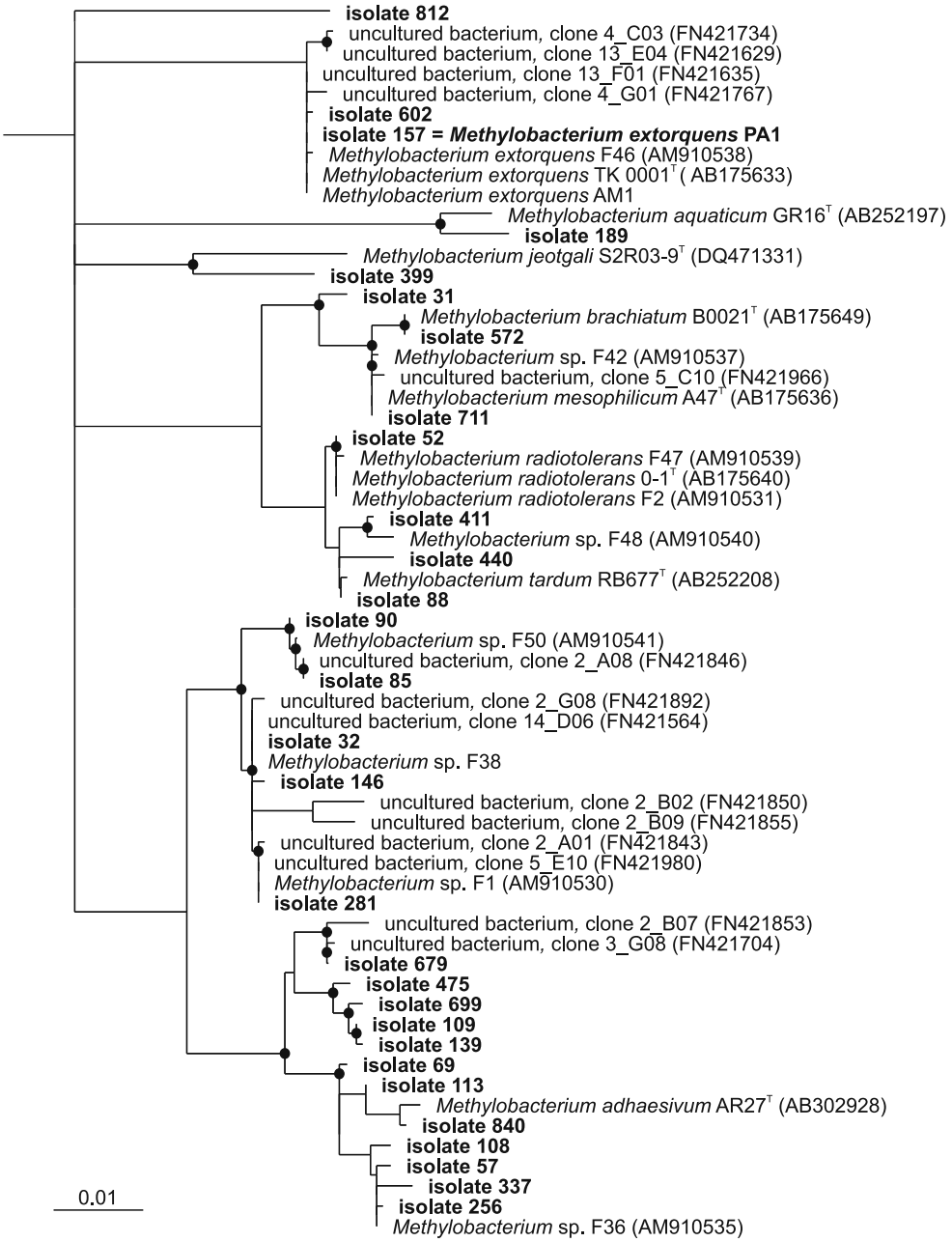

Figure 1 16S rRNA gene sequence based tree showing the phylogenetic placement of the isolates obtained in this study in relation to *Methylobacterium* type strains and to sequences detected in the phyllosphere by cultivation-dependent and independent methods. The tree was calculated based on 1,383 aligned nucleotide positions using the Treepuzzle algorithm with the HKY evolutionary model [18] and 25,000 puzzling steps. Branches that were retrieved in more than 80% of all trees are marked by *black circles*, while branches with <50% retrieval are shown as *multifurcations*. These multifurcations reflect the major differences observed between a distance-method-based tree and a maximum likelihood tree. The outgroup (not shown) consisted of sequences from five different alphaproteobacterial genera (*Albibacter methylovorans*, AF273213; *Methylosulfomonas methylovora*, U62893; *Afipia felis*, AF338177; *Bradyrhizobium japonicum*, BA000040; *Rhodospirillum rubrum*, X87278). The *bar* indicates 0.01 change per nucleotide position

600 16S rRNA gene sequences clustering within the genus were considered. Sequences were taken from the SSU Ref database (version 100) of the SILVA rRNA database project, which contains only high quality nearly full length sequences [47]. Strains that were detected in association with plants represented 25% of all available sequences and were present in 44 out of 104 different sequence clusters (42%; Fig. S1). These sequence clusters were formed based on a 99% sequence identity level. The distribution of plant-associated strains within the genus was not restricted to a few distinct lineages; in contrast they were found across the whole phylogenetic tree, indicating that diverse members and not just a distinct lineage within the genus *Methylobacterium* have capabilities for successful plant colonization. However, some members may be more successful than others and may therefore be more frequently detected in association with plants than others. This may serve as one explanation for the observation that the isolates of this study reflected only part of the *Methylobacterium* diversity that is seen on various plants all over the world. Another possible explanation for this discrepancy is given by the fact that plants from a restricted geographical area, a restricted set of ecosystems and from similar climatic regions were analyzed in this study.

Competitiveness of Diverse Methylobacterium Strains in the Phyllosphere of A. thaliana Three different competition experiments were performed to study the colonization

Table 1 Presence of 16S rRNA genotypes among the different sampling sites

Isolate	A1	A2	A3		A4		A5	M1				M2			M3			
	Leaf	Leaf	Leaf	Soil	Leaf	Soil	Leaf	Leaf	Root	Soil	Air	Leaf	Soil	Air	Leaf	Root	Soil	Air
31	+	+	+				+	+				+		+	+			+
32	+	+	+		+		+				+	+	+	+	+			+
52			+															
57			+															
69	+	+	+	+	+	+	+	+				+	+	+	+			+
85	+				+		+								+			
88							+								+			
90							+											
108	+	+	+		+		+	+	+		+	+		+	+			+
109	+	+	+					+				+			+			
113	+													+				
139		+	+				+	+				+			+			
146			+															
157	+				+		+	+	+	+					+	+		+
189					+			+				+						
256			+				+	+			+	+		+	+		+	
281	+	+	+		+		+	+			+				+	+	+	+
337		+	+				+	+										
399							+											
411			+															
440							+											
475														+				
572															+			
602																+		+
679	+							+										
699												+						
711												+						
812	+			+														
840															+			

Each genotype is represented by one isolate in this table. A1–A5 indicate the different sampling sites that harbored *A. thaliana* populations; M1–M3 sites with *M. truncatula* populations

efficiency of *Methylobacterium* strains in the phyllosphere and are described in this and the following two sections. In a first competition experiment, the competitiveness of a representative subset of 174 isolates (Table S1) was studied on the model plant *A. thaliana* Col-0. We maximized strain diversity in this first experiment and included isolates from diverse plant species, though the selection was dominated by isolates from *A. thaliana*. The selection of isolates was essentially made based on the BOX-PCR pattern. (Exemplarily, BOX-patterns of isolates obtained from one of the sampling sites are shown in Fig. S2.) BOX-PCR enables differentiation of isolates to the strain level [48] and allowed identification of genetically (nearly) identical isolates. Isolates with highly similar BOX-patterns may in fact represent the same strain that multiplied within the sampling site and was recovered several times. Only one isolate was included in the competition experiment in cases where several isolates with highly similar BOX-patterns were obtained from the same sampling site, so that we assume that only different strains were tested. In the competition experiments strain differentiation was based on ITS1 sequence analysis. The 174 selected isolates represented 110 different ITS1 sequence types and included 18 strains with multiple different *rrn* operons.

The competitiveness of each strain was tested in five-strain-mixtures based on two different strain combinations per strain. These mixtures were applied to sterilized *A. thaliana* seeds during sowing. After 3 weeks of plant growth in the greenhouse, the dominating strains in the plant phyllosphere were identified. Ten strains were highly

Table 2 Concordance of ARISA fragments detected in isolates and during the cultivation-independent analysis of the leaf samples[a, b]

Site	% of the community seen in the cultivation-independent analysis that can be explained with isolates from[c]		Number of samples taken at that site	% of isolates whose ARISA pattern was seen in the cultivation-independent analysis in samples collected at		Number of isolates[d]
	The same site	Any site		The same site	Any site	
A1	40±13	90±5	22	92	100	48
A2	76±13	89±12	29	99	99	70
A3	58±17	95±5	37	100	100	83
A4	65±11	89±8	28	90	100	72
A5	83±9	95±5	44	83	99	109
M1	80±19	97±6	19	95	97	37
M2	94±10	99±2	23	55	99	108
M3	83±18	98±4	27	96	100	103

[a] Cultivation-independently retrieved data were taken from the study of Knief et al. 2010 [28]

[b] A one-basepair shift was allowed for the assignment of peaks between cultivation-independent data and isolate data, as isolates were only analyzed once and the obtained peak size was rounded to the nearest integer

[c] The relative abundance of those ARISA fragments in the cultivation-independently retrieved pattern that were seen in an isolate was summarized for each sample. Mean values±one standard deviation calculated from all individual samples that were collected at the respective sites are presented

[d] Isolates for which an ARISA pattern was obtained

competitive, they dominated in both strain combinations. Further 34 strains dominated in one of the combinations, and 61 strains were detected in one or in both combinations, but not as the most abundant *Methylobacterium* strain. The 44 strains that dominated in at least one of the two strain combinations were obtained from different sampling sites and diverse plant species. The fact that isolates from a certain site or from *A. thaliana* plants did not perform better during competition on *A. thaliana* than isolates from other sites or plant species is congruent with the observation that *Methylobacterium* strains do not colonize plants in a highly plant species-specific manner [28]. In contrast, competitiveness of the strains was linked to their phylogenetic placement at 16S rRNA gene and ITS1 sequence level. The distribution of competitive and non-competitive strains within the diverse sequence clusters deviated significantly ($P<0.001$) from an equal distribution. The majority of the highly competitive strains were *M. extorquens* strains (Fig. 2). In fact, all tested *M. extorquens* strains except isolate 347 could be rediscovered after growth under competitive conditions, even though not all dominated a competition. Other competitive colonizers were related to the type strain *M. adhaesivum* AR27^T. These include isolates of the 16S rRNA genotypes represented by strains 32, 85, 256, and 281. However, not all of the different ITS1 sequence clusters that represented the 16S rRNA genotype of isolate 32 contained competitive strains. Remarkably, isolate 88, which is most closely related to *M. tardum* RB677^T, was so competitive under the given conditions that no other *Methylobacterium* strain could be detected on the individual plants using our analysis method.

Competitiveness of Methylobacterium Strains Under Different Plant Growth Conditions A second competition experiment was performed based on a subset of 17 strains to evaluate the reproducibility of the observed results under different growth conditions, i.e., on plants grown in peat pellets under greenhouse conditions (as before) and plants grown under gnotobiotic conditions on a synthetic medium. The tested strains were selected from those used in the first competition experiment, mainly including the competitive ones, which also allowed for a more detailed evaluation of the competitiveness of these strains between each other. Moreover, the well-studied laboratory strain *M. extorquens* AM1 [55, 62] and two other isolates from plants, *M. radiotolerans* F2 and *M. mesophilicum* F42, which were isolated in a previous study [27], were included due to the frequent detection of closely related strains in the phyllosphere in other studies (Fig. S1) [e.g., 2, 4, 5, 7, 21, 22, 27] and the very good performance of the closely related isolate 88 in the first competition experiment. The competitiveness of the analyzed strains was similar under both plant growth conditions with respect to the most competitive (isolate 88, *M. radiotolerans* F2 and *M. mesophilicum* F42) and the least competitive strains (isolates 32 and 337 and *M. extorquens* AM1; Table 3). The stability in the colonization efficiency under the different plant growth conditions suggests that the altered a-/biotic factors such as humidity, plant nutrition or the presence of other heterotrophic bacteria did not strongly affect the competitiveness of those strains that represented very distinct species. However, the performance of closely related strains, e.g., of most *M. extorquens* strains, was more variable under the two conditions (Table 3).

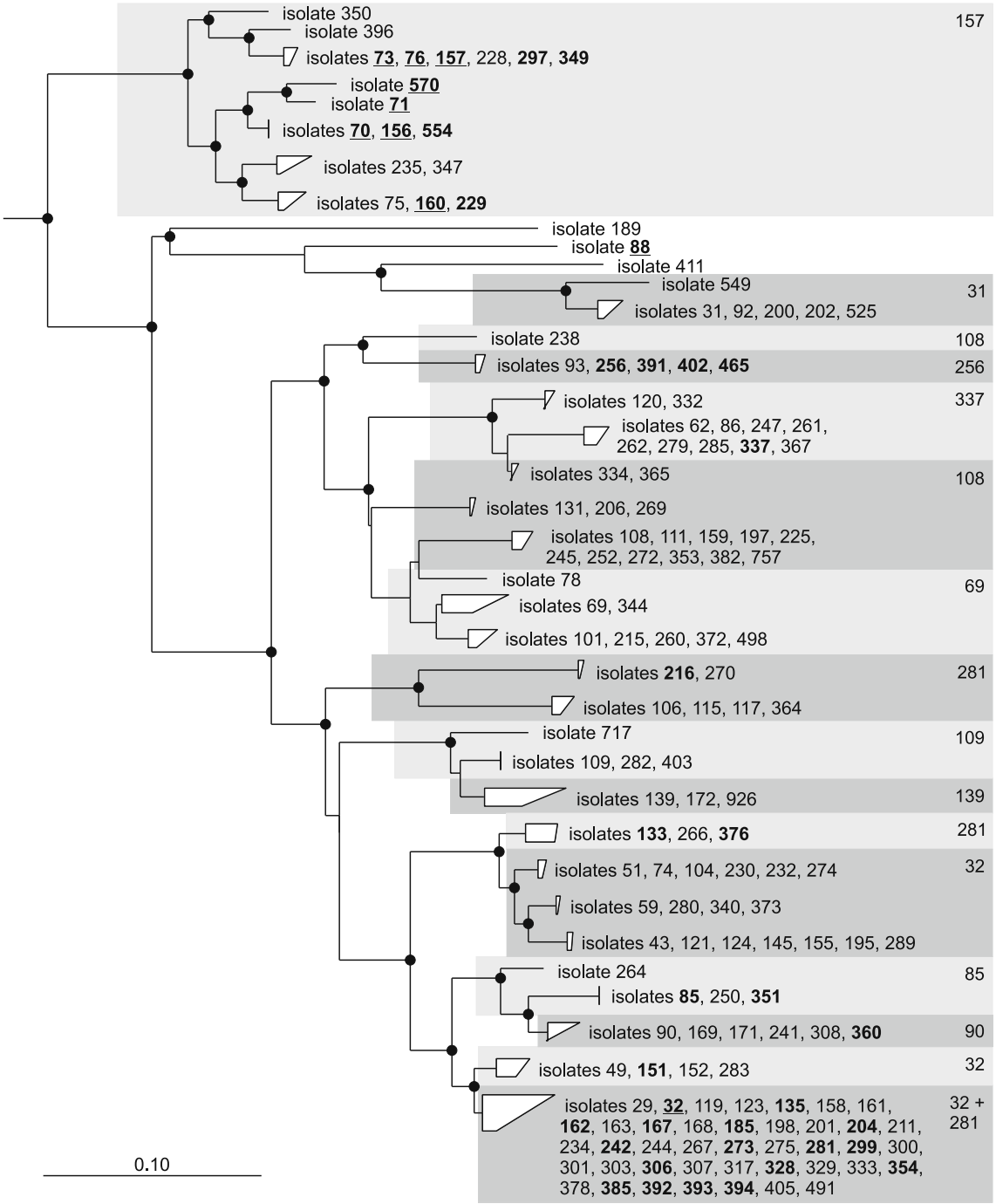

Figure 2 Phylogenetic tree summarizing the results of the first competition experiment. The tree was calculated based on ITS1 sequences of the *Methylobacterium* isolates used in the competition experiment. The sequence clusters *shaded in gray* represent isolates belonging to the same 16S rRNA genotype as indicated by the *number in the upper right corner*. Competitive strains that dominated in one strain combination are highlighted in *bold*, very competitive strains that dominated in both strain combinations are additionally *underlined*. Eighteen strains with multiple different ITS1 sequences that were included in the competition experiment are not shown. Two of them, isolates 170 and 357, which have identical 16S rRNA gene sequences as isolates 281 and 32, respectively, were identified as competitive strains. The tree was calculated based on 1,228 aligned nucleotide positions using the neighbor-joining algorithm with a Jukes-Cantor correction. Bootstrap values were calculated based on 1,000 replicates; values >80% are indicated by *black dots.* Sequence clusters with a sequence dissimilarity <5% were grouped when possible. The *bar* indicates 0.1 change per nucleotide position. Sequences of other alphaproteobacterial genera (*Rhodopseudomonas palustris*, AF338178; *Bradyrhizobium japonicum*, BA000040; *Afipia felis*, AF338177) were used to define the outgroup (not shown)

Table 3 Competitiveness of *Methylobacterium* strains on *A. thaliana* plants grown under different conditions as evaluated in the second competition experiment

Strain	16S rRNA genotype	Performance (rank no.)[a]		Abundance upon single inoculation, gnotobiotic ($\times 10^8$ cells per g leaf material)
		Greenhouse	Gnotobiotic	
F2	*M. radiotolerans*	2	1	3.27
F42	*M. mesophilicum*	3	4	2.82
88	88	1	2	1.95
570	*M. extorquens* 157	9	13	1.79
235	*M. extorquens* 157	7	12	1.71
90	90	14	3	1.41
306	281	12	16	1.34
156	*M. extorquens* 157	6	5	1.27
160	*M. extorquens* 157	11	7	1.15
229	*M. extorquens* 157	10	8	1.13
70	*M. extorquens* 157	8	9	1.10
157	*M. extorquens* 157	4	6	1.00
71	*M. extorquens* 157	5	10	0.92
32	32	15	14	0.88
337	337	16	15	0.77
256	256	13	11	0.48
AM1	*M. extorquens* 157	17	17	0.24

[a] To compare the relative performance of the strains, a ranking of the four strains tested in each combination was deduced from the replicate suspensions. Most strains were tested in five to seven different combinations (detailed results presented in Table S2). This ranking was used to calculate the mean ranking of a strain

The plant colonization efficiency of the strains was further characterized in inoculation experiments under non-competitive conditions. The three strains that reached the highest cell numbers ($1.95–3.27 \times 10^8$ CFU per gram of leaf fresh weight) in these single inoculation experiments were the more competitive ones when applied to the plant in a mixture, while those colonizers with the lowest numbers (*M. extorquens* AM1, isolates 256, 337 and 32; $2.4–8.8 \times 10^7$ cells per gram of leaf fresh weight) were less competitive (Tables 3 and S2). These data suggest that the extent to which the strains are able to profit from the resources of a plant is an important factor in determining the competitiveness of a strain.

A comparison of cell numbers from gnotobiotically grown plants inoculated with only one strain and plants colonized by a mix of four strains revealed that the plants inoculated with the strain mixture harbored on average more cells (about threefold) than plants inoculated with a single strain (Table 3 and S2). When comparing the cell number determined on plants harboring a mix of four strains with the number reached by that specific strain among the four in the mix that was the most efficient colonizer upon single inoculation a significant 1.7-fold increase was observed (one-sided paired samples *t* test applied on log-transformed data from mixes versus the respective most efficient single colonizer strain: $P=0.012$). This indicates that the different strains, or at least some of those that were combined in a mixture, must have occupied slightly different niches, which enabled them to compete successfully with each other and to coexist.

Competitiveness of Methylobacterium Strains Isolated in Other Studies Finally, the colonization efficiency of strains isolated from various habitats and in different studies was compared to the performance of the strains obtained in this study (Table 4). The selection included all currently genome-sequenced *Methylobacterium* strains, as these are good candidates for future targeted studies, e.g., to identify traits for competitive phyllosphere colonization. All of the strains were tested against a competitive *M. extorquens* strain from this study, isolate 157 (now designated as *M. extorquens* PA1, see below). The competitiveness of strains that were isolated from plants was variable: while *M. radiotolerans* 0-1^T performed very well, *Methylobacterium populi* BJ001^T was not competitive. The root nodulating strains *Methylobacterium nodulans* ORS 2060^T and *Methylobacterium* sp. 4-46 also were not competitive in the phyllosphere. This is not surprising, as these strains, which belong to a monophyletic cluster of nodulating strains within the genus, have developed a specialized lifestyle: they undergo a close symbiotic interaction with specific host plants [24, 40, 57] and have most probably adopted a rhizospheric and endosymbiotic lifestyle. The majority of the *M. extorquens*

Table 4 Performance and origin of strains analyzed in the third competition experiment

No.	Strain	Isolation source	References	In competition with isolate 157 and strain no.	Competition compared to isolate 157[a]
1	*M. extorquens* AM1 [b]	Airborne contaminant of a methylamine containing medium (UK)	[42]	8, 9 and 12 [c]	–
2	*M. extorquens* TK0001^T (DSM 1337)	Soil (Poland)	[60]	6	+
3	*M. extorquens* F (DSM13060)	Tissue culture of *Pinus sylvestris* (Finland)	[44]	13	=
4	*M. extorquens* SM5	Beer bottling plant (Germany)	Unpublished [d]	14	=
5	*M. extorquens* SM14	Beer bottling plant (Germany)	Unpublished [d]	15	-
6	*M. extorquens* CM4 [b, e]	Soil at a petrochemical factory (Russia)	[8]	2	=
7	*M. extorquens* DM4 (DSM 6343) [b, f]	Soil from a treatment plant for halogenated hydrocarbon waste (Germany)	[30], Gälli 1986 [g]	16	=
8	*M. thiocyanatum* ALL/SCN-P^T (DSM 11490)	Soil of root ball of *Allium aflatunense* (UK)	[63]	1	+
9	*M. hispanicum* GP34^T (DSM 16372)	Drinking water of municipal water supply (Spain)	[13]	1	++
10	*M. aquaticum* GR16^T (DSM 16371)	Drinking water of municipal water supply (Spain)	[13]	11	–
11	*M. populi* BJ001^T [b]	Tissue of *Populus deltoides* x *nigra* DN34 (USA)	[61]	10	–
12	*M. radiotolerans* 0-1^T (DSM 1819) [b]	Rice grains (Japan)	[22]	1	++
13	*M. fujisawaense* 0-31^T (DSM 5686)	Unknown (Japan)	[16]	3	++
14	*M. mesophilicum* A 47^T (DSM 1768)	Leaves of *Lolium perenne* (UK)	[5]	4	=
15	*M. rhodinum* TK0010^T (DSM 2163)	Rhizosphere of *Alnus* (Germany)	[19]	5	-
16	*M. podarium* FM4^T (DSM 15083)	Human foot (UK)	[3]	7	-
17	*M. nodulans* ORS2060^T [b]	Nodules of *Crotalaria podocarpa* (Senegal)	[52]	18	–
18	*M.* sp. 4-46 [b, h]	Nodules of *Lotononis bainesii* (South Africa)	[40]	17	–

All strains were tested in competition with isolate 157 (=*M. extorquens* PA1) plus a third strain

[a] Symbols: (++) strain clearly dominated the competition, (+) strain was better than isolate 157, (=) strain was as competitive as isolate 157, (-) strain was less competitive than isolate 157, (–) strain was very weak and thus not detectable

[b] Genome sequenced

[c] *M. extorquens* AM1 was tested in three different combinations consisting of isolate 157 plus one of the listed strains

[d] Strain was kindly provided by André Lipski (Universität Bonn, Germany)

[e] Synonymous with *M. chloromethanicum* CM4 [26]

[f] Synonymous with *M. dichloromethanicum* DM4 [26]

[g] Gälli R (1986) Optimierung des mirkobiellen Abbaus von Dichlormethan in einem Wirbelschicht-Bioreaktor. ETH Zurich, Dissertation

[h] This strain is also referred to as CB376 [11]

strains performed as well as isolate 157, regardless of their isolation source. This confirms that the colonization efficiency of *Methylobacterium* strains on *A. thaliana* is in general correlated with their phylogenetic placement.

Identification of Representative Model Strains and Characterization of M. extorquens PA1 Based on the results of the competition experiments, strains with different colonization efficiencies on *A. thaliana* plants were selected and will serve as models for future research to address open questions in this plant–microbe association. Among the genome-sequenced strains, *M. radiotolerans* 0-1^T and *M. populi* BJ001^T represent suitable models. Both strains were isolated from plants (Table 4). While *M. radiotolerans* 0-1^T represents a group of phylogenetically related, very competitive *A. thaliana* phyllosphere colonizers, consisting of the species *M. radiotolerans*, *M. mesophilicum,* and *M. tardum*, *M. populi* BJ001^T can serve as model for a weak colonizer of *A. thaliana.* As several competitive strains were found within the species *M. extorquens*, a further representative strain was selected from this species. We chose isolate 157, now designated *M. extorquens* PA1 (PA = phyllosphere of *Arabidopsis*), because it was isolated from the leaves of the model plant *A. thaliana* and is very closely related to the well-studied genome-sequenced model organism *M. extorquens* AM1 based on 16S rRNA gene and ITS1 sequence data (100% and 95.9% sequence identity, respectively).

As a basis for future studies, *M. extorquens* PA1 was characterized in more detail. As expected, it has morphological and physiological characteristics similar to other *M. extorquens* strains (Table S3, Table S4). Carbon source utilization was studied using Biolog plates, which test for respiratory activity upon carbon consumption. *M. extorquens* PA1 showed activity in the presence of several C5-sugars and diverse organic acids, but not in the presence of amino acids or nucleosides (Table S4). Acyl-homoserine lactone (HSL) production was analyzed as described by Penalver et al. [43]. Bioassay tests with *Pseudomonas putida* F117, which responds to the presence of long-chain HSLs (C_{10}-HSL, C_{12}-HSL, and C_{14}-HSL), were positive. The presence of the long-chain HSLs C12:1, C14:1, C16:1, and C14:2 was confirmed by liquid chromatography–mass spectrometry of extracts from cells grown in the presence of methanol and succinate. The synthesis of long-chain HSLs has also been shown in other *Methylobacterium* strains [43, 45]. Short-chain HSL (C_6-HSL and C_8-HSL) production was not detected in *M. extorquens* PA1, although this trait is known for some other *Methylobacterium* strains [43, 46]. A draft version of the complete genome sequence of *M. extorquens* PA1 is publicly available and its annotation is in progress (Marx et al., in preparation). The strain has been deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ).

Conclusion

Plant colonization is an ability that is apparently dispersed among diverse members of the genus *Methylobacterium*. These members must have developed adaptations that allow them to live in the phyllosphere, where they are exposed to temperature shifts, desiccation, nutrient limitation, and UV irradiation [34]. Based on the results of the competition experiments phylogenetically closely related *Methylobacterium* strains often colonize plants with similar efficiencies, whereas strains belonging to different genera show clear differences during competition. This suggests that the physiology of the strains may be an important factor in determining the plant-colonization efficiency. This hypothesis is supported by the observation that the strains that were most successful under competitive conditions also colonized the plants with higher cell numbers under non-competitive conditions. The distinct properties of different *Methylobacterium* species can be expected to enable at least some members of the genus to colonize a certain plant under a given condition; and by occupying slightly different niches, a complex *Methylobacterium* community can be established in the phyllosphere. This may partly explain the consistent success of the genus *Methylobacterium* in the phyllosphere of diverse plants. The identification of strains with different colonization efficiencies serves as a basis for future studies aiming at the identification of factors that affect the *Methylobacterium* community composition.

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